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The influence of pH on the G-quadruplex binding selectivity of perylene derivatives

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Abstract—Three new perylene derivatives with branched ionizable side chains were synthesized, and their G-quadruplex binding specificities were compared by spectroscopic and electrophoretic analysis with two well-studied G-quadruplex ligands: PIPER and $TmPyP_4$. The value of pH and consequent charge formation and self-aggregation of these perylene derivatives influences not only the type of G-quadruplex formation, but also the G-quadruplex binding selectivity.

G-rich DNA sequences can adopt a special class of DNA structure called a G-quadruplex, which comprises a stack of G-tetrads, the planar association of four guanines in a cyclic Hoogsteen hydrogen bond. G-quadruplex ligands are proposed to be selective anticancer agents by acting as telomerase inhibitors[1] and/or transcriptional repressors of c-*MYC* oncogene.[2] However, one problem that faces many G-quadruplex ligands is non-specific cytotoxicity, which is believed to arise from their interaction with duplex DNA.[1b,3] Ideal G-quadruplex ligands, therefore, should bind selectively to their target and have little interaction with duplex DNA. Studies regarding G-quadruplex binding selectivity are essential to the development of G-quadruplex ligands for therapeutic use.

Among several classes of G-quadruplex ligands, perylene is one of the most widely studied.[4] The perylene derivatives, including the prototype, PIPER, have been well characterized with regard to their G-quadruplex-bound structure,[4a] G-quadruplex induction,[4b,c,h,i] G-quadruplex binding selectivity,[4d–i] and telomerase inhibition.[4h,i] The binding specificity of perylene toward G-quadruplex DNA is exemplified by the cleavage experiment of perylene-EDTA·Fe(II). This complex, upon initiating hydroxyl radical production, cleaved preferentially at the G-quadruplex region, with little effect on the duplex region of the same DNA molecule.[4d] To develop this class of molecule into useful therapeutic agents, studies of G-quadruplex binding selectivity are essential.

Here, we report the synthesis and characterization of three new perylene derivatives (see Fig. 1): P-GLU, P-HIS, and P-TRIS,[5] and the influence of pH on the solubility and G-quadruplex binding selectivity of these molecules in comparison with the two well-studied G-quadruplex ligands: PIPER and $TmPyP_4$. The four perylene derivatives possess side chains that are ionizable with systematically varying charges as a function of pH (see Fig. 2).[6] Over the entire range of buffers used in this study (pH 5–9), P-GLU is negatively charged, while PIPER and P-TRIS are positively charged. P-HIS, however, is neutral at lower pH (~5) but is negatively charged at higher pH. Therefore, the effect of neutral and negatively charged P-HIS toward G-quadruplex selectivity can be compared directly without considering structural differences. The solubility profiles of these perylene derivatives are shown in Table 1.

We first determined the binding specificity of the perylene derivatives with various preformed DNA structures by spectrophotometry. Each perylene derivative (40 μM) was incubated with each preformed DNA

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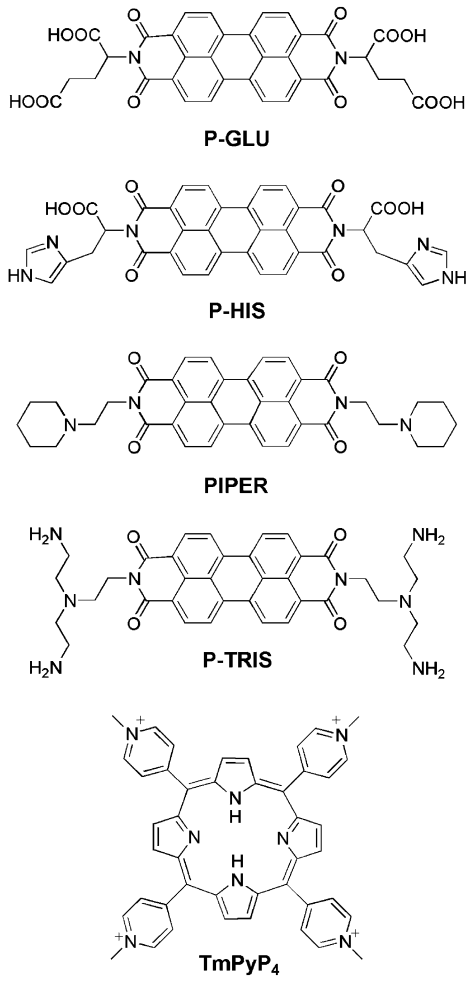

Figure 1. Structures of perylene derivatives and $TmPyP_4$.

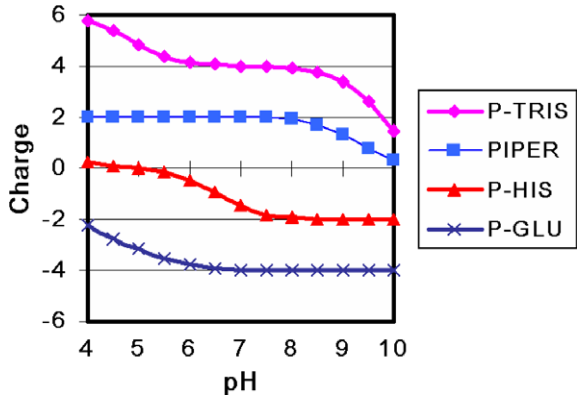

Figure 2. Calculated charges of perylene derivatives as a function of pH.

Table 1. Solubility profiles of perylene derivatives

pH	5*	6**	7**	8**	9***
P-GLU	+	+	+	+	+
P-HIS	–	–	±	+	+
PIPER	+	+	–	–	–
P-TRIS	+	–	–	–	+

Solubility was recorded after dispersion of the compound (50 μM) in the designated buffer for seven days. Symbols: (+) soluble, (±) partially soluble, (–) precipitate, (*) potassium acetate buffer, (**) potassium phosphate buffer, and (***) Tris–HCl buffer.

structure[7] (20 μM): single-stranded DNA (ss-DNA, 24A4), double-stranded DNA (ds-DNA, 12D), G3-quadruplex DNA (G3-DNA, 24G3), or G4-quadruplex DNA (G4-DNA, 24G4), in a designated buffer containing 100 mM KCl for 18 h, and absorption spectra between 400 and 600 nm were recorded (Fig. 3). The sequences of all oligonucleotides used in this study are shown in Table 2.

The spectra of P-GLU with the various DNA substrates show no significant changes from the spectrum of P-GLU alone, indicating that P-GLU fails to interact with any of the DNA structures. Considering that P-GLU is negatively charged and dissolves well at all buffers used, it is plausible that the branched negatively charged side chains of P-GLU prevent the interaction with DNA via electrostatic repulsion with the phosphate backbone of DNA.

The spectra of P-HIS with the various DNA substrates show pH-dependent and substrate-dependent interactions. At pH 6, spectra of P-HIS with ss-DNA, ds-DNA, or P-HIS alone are almost flat across all wavelengths due to aggregation, indicating that P-HIS fails to interact with these DNA substrates. In contrast, the spectra of P-HIS with G-quadruplex DNAs (both G3-DNA and G4-DNA) show a significant increase in absorption intensity, especially at 550 nm, indicating strong interaction of P-HIS with these DNA substrates. When the pH is increased, the specific interaction of P-HIS with the G-quadruplex DNAs diminishes, which is reflected by the small differences in spectral intensity. The absorption intensity of P-HIS alone increases with pH, consistent with the enhanced solubility of this compound at higher pH values.

Considering the charge of the P-HIS side chains, the two carboxylic groups are deprotonated at pH 6, and some of the imidazole nitrogens are protonated. The average charge of P-HIS at pH 6 is −0.45.[6] When the pH of the buffer increases, P-HIS becomes more negatively charged (pH 7 = −1.49 and pH 8 = −1.93),[6] and the specific interactions of P-HIS with the G-quadruplex DNAs are less, which is again reflected by the small differences in spectral intensity.

The spectra of PIPER with the various DNA substrates also show pH-dependent and substrate-dependent interactions. The binding preference follows the order G4-DNA ≅ G3-DNA > ds-DNA > ss-DNA. When the pH is increased, self-aggregation of PIPER increases, as illustrated by the general decline in absorption intensity. The G-quadruplex selectivity also increases with increasing pH, as illustrated by the increasing differences in spectral intensity for the various DNA substrates.

The spectra of P-TRIS with the various DNA substrates show patterns similar to those of PIPER. Although P-TRIS aggregates at all pH values used, it appears that the aggregation of P-TRIS is enhanced at higher pH. The binding preference follows the order G4-DNA > G3-DNA > ds-DNA > ss-DNA. Compared to PIPER, the G-quadruplex binding selectivity is less,

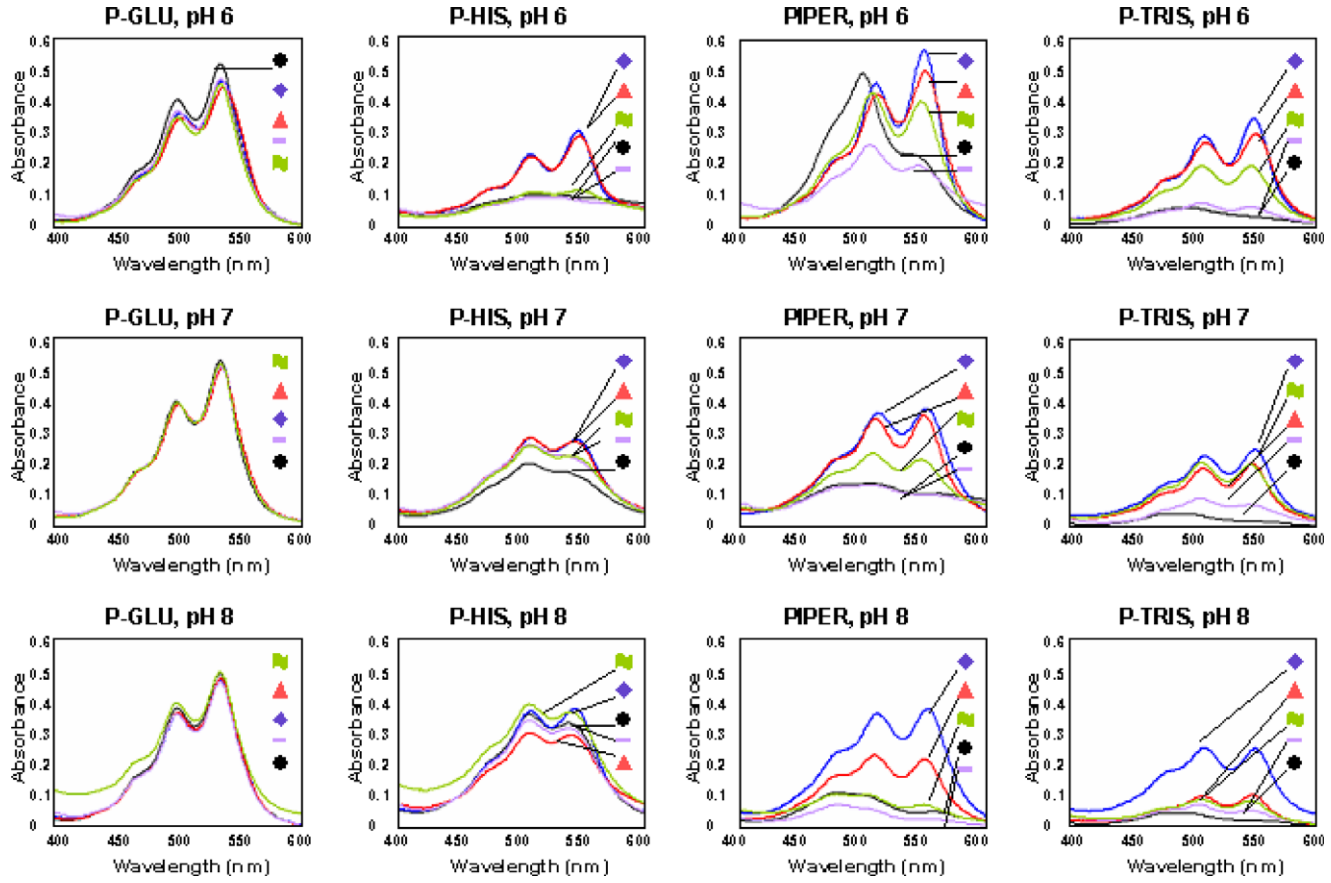

Figure 3. Visible absorbance spectra of perylene derivatives in the absence (●) and presence of preformed DNA structures: G4-DNA (◆), G3-DNA (▲), ds-DNA (≈), and ss-DNA (–), in 10 mM phosphate (pH 6 and 7) or Tris–HCl (pH 8), and 100 mM KCl, after 18 h of incubation.

Table 2. Oligonucleotides used in this study

Names	Sequences
12D	CGC GAA TTC GCG
24A4	$(TTA\ AAA)_4$
24G3	$(TTA\ GGG)_4$
24G4	$(TTG\ GGG)_4$
32C3	AA $(CCC\ TAA)_4$ CTA TCT
32G3	AGA TAG $(TTA\ GGG)_4$ TT
NHE27C	CCT TCC CCA CCC TCC CCA CCC TCC CCA
NHE27G	TGG GGA GGG TGG GGA GGG TGG GGA AGG

which might arise from the greater positive charges of the P-TRIS ligand.

Altogether, the data from the spectrophotometric studies suggest that the branched negatively charged perylenes, such as P-GLU and P-HIS at pH 8, which are dissolved well in the buffer, fail to interact with any of the DNA structures. However, the two previously reported negatively charged perylenes, perylene-EDTA and Tel12, which were able to aggregate, bound preferentially to G-quadruplex DNA.[4d,f] The closely branched structure of P-GLU and P-HIS and the negative charges might prevent them from self-aggregation and DNA binding.

In contrast, the zwitterionic form of P-HIS (at pH 6) and the positively charged perylenes, such as PIPER and P-TRIS, prefer binding to G-quadruplex DNAs. The G-quadruplex binding selectivity is best with the zwitterionic form of P-HIS. When the positive charges increase, as with PIPER and P-TRIS, the G-quadruplex selectivity is diminished.

The effects of pH on G-quadruplex binding and/or formation of human telomeric DNA by perylene derivatives or $TmPyP_4$, a well-characterized G-quadruplex ligand,[2d,8] were further analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE). Figure 4 shows that the patterns of radiolabeled G-strands of the telomeric sequence (32G3) are constant with increasing concentration of P-GLU and P-HIS at all pHs examined, indicating that P-GLU and P-HIS fail to interact with the G-quadruplex formed from this strand. For P-GLU, the data from the gel experiments support the data from spectrophotometry, where P-GLU interacts with none of the types of DNA at all pHs examined. For P-HIS, the spectrophotometric data show that P-HIS binds preferentially to G-quadruplex at low pHs. The gel data, however, fail to support this trend for this oligonucleotide. Nevertheless, as shown below, P-HIS binds to G-quadruplex DNA in a pH-dependent manner with an oligonucleotide from c-*MYC* promoter.

PIPER shows pH-dependent and concentration-dependent binding to G-quadruplex. It preferentially

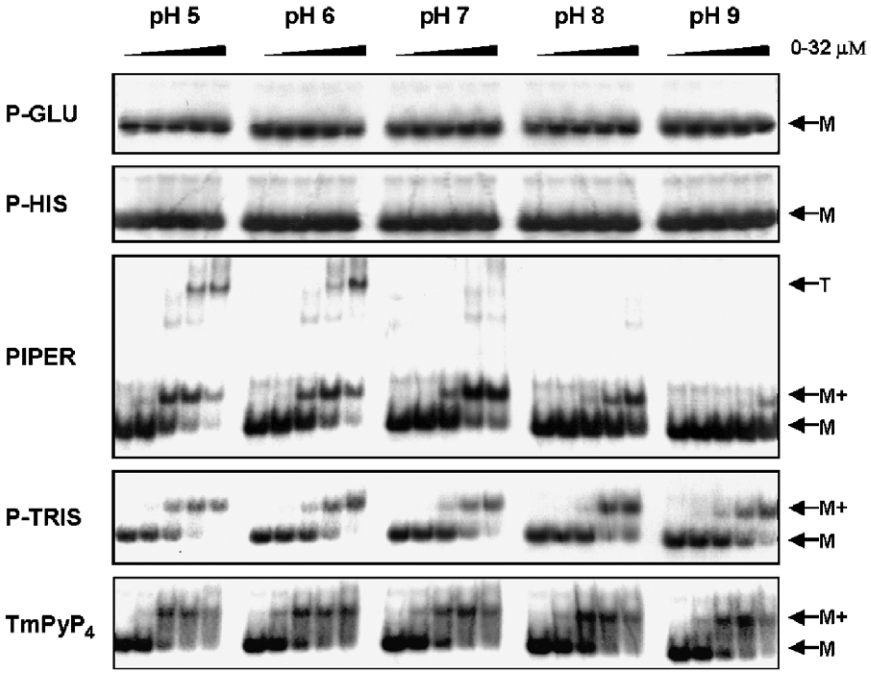

Figure 4. Influence of pH on G-quadruplex binding/formation. The telomeric oligonucleotide (32G3, 2 μM) was mixed with each compound (0, 4, 8, 16, and 32 μM) in 10 mM buffer containing 100 mM KCl (pH 5 = potassium acetate buffer, pH 6–8 = potassium phosphate buffer, and pH 9 = Tris–HCl buffer). The mixture was heated to 95 °C for 5 min and then incubated at 55 °C for 10 h in a thermocycler before cooling to 4 °C. The samples were separated by electrophoresis at 4 °C in a 16% non-denaturing polyacrylamide gel; both gel and TBE buffer were supplemented with 50 mM KCl. Bands are identified as monomeric G-quadruplex (M), ligand bound monomeric G-quadruplex (M+), and tetramolecular G-quadruplex DNA (T).

binds to monomeric G-quadruplex (M+) at low concentration, but facilitates the formation of tetrameric G-quadruplex (T) at high concentration. The variation with pH might reflect the availability of PIPER in solution. As stated earlier, the aggregation of PIPER is enhanced at high pH. Consequently, as the pH is increased, soluble PIPER is less available to bind to G-quadruplex. Therefore, the gel pattern observed for higher concentrations of PIPER at higher pH resembles that of lower concentrations at lower pH. The M and M+ bands are both monomeric G-quadruplex, as verified by DMS methylation protection assay (Supplementary data S9). We found that the M+ band was bound with PIPER, while the M band was not (Supplementary data S10).

P-TRIS and $TmPyP_4$ both show concentration-dependent binding to G-quadruplex. The influence of pH on G-quadruplex binding is, however, not as pronounced as with PIPER. Moreover, neither P-TRIS nor $TmPyP_4$ facilitates the formation of tetrameric G-quadruplex like PIPER does.

To assess the binding preference between double-stranded DNA and G-quadruplex DNA of the perylene derivatives and $TmPyP_4$, we employed a known duplex/G-quadruplex competition assay.[4c] Figure 5 shows that without ligand, these oligonucleotides prefer to form duplex in all buffers used. However, when they were incubated with PIPER or P-TRIS, the G-strand forms monomeric G-quadruplex (M+) in a concentration-dependent manner. PIPER can also induce tetrameric G-quadruplex, while P-TRIS cannot. In contrast to PIPER and P-TRIS, $TmPyP_4$ binds to double-stranded DNA (DS) in a concentration-dependent manner without inducing G-quadruplex formation.

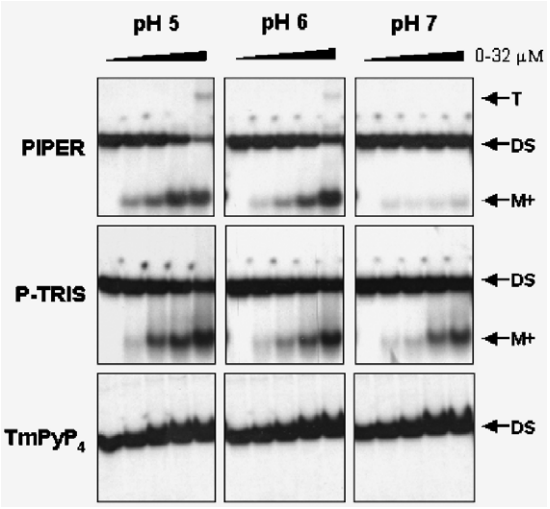

Figure 5. Duplex/G-quadruplex competition assay with telomeric sequence. The duplex (32G3/C3, 2 μM) was mixed with PIPER, P-TRIS, or $TmPyP_4$ (0, 4, 8, 16, and 32 μM) in 10 mM buffer containing 100 mM KCl (pH 5 = potassium acetate buffer, pH 6–7 = potassium phosphate buffer). The mixture was heated to 95 °C for 5 min and then incubated at 55 °C for 10 h in a thermocycler before cooling to 4 °C. The samples were separated by electrophoresis at 4 °C in a 16% non-denaturing polyacrylamide gel containing 50 mM KCl in both gel and electrophoresis buffer. Bands are identified as ligand-bound monomeric G-quadruplex (M+), double-stranded DNA (DS), and tetrameric G-quadruplex DNA (T). Only the results from the radiolabeled G-strand are shown.

P-GLU and P-HIS cause no change in the pattern of the duplex at any concentration or at any pH (data not shown).

The change in pH affects G-quadruplex/duplex formation differently with each ligand. For PIPER, increasing pH reduces the formation of G-quadruplexes. As we mentioned earlier, increasing pH reduces the availability of PIPER in solution due to the precipitation of PIPER at higher pH. The results here confirm the results from the previous G-quadruplex formation/binding assay (Fig. 4). For P-TRIS and $TmPyP_4$, the change of pH slightly affects the G-quadruplex induction of P-TRIS but fails to influence the gel pattern of $TmPyP_4$ at all.

From the above gel experiments, we explored the binding of the perylene derivatives and $TmPyP_4$ with telomeric oligonucleotide. To study the influence of DNA sequence, we tested the compounds with the DNA sequence from c-*MYC* promoter. The G-rich strand of the 27-mer c-*MYC* promoter forms two intramolecular G-quadruplex structures: the kinetically favored chair conformer and the thermodynamically favored basket conformer. The chair conformer can form instantly in solution with or without KCl and is the major product even after incubation at 37 °C for 48 h.[2d] Previous reports have shown the PIPER-induced G-quadruplex formation of this sequence in the duplex/G-quadruplex competition assay,[4c] and $TmPyP_4$-stabilized G-quadruplex formation of this sequence in the polymerase stop assay.[2d]

Figure 6 indicates that the G-rich strands of c-*MYC* promoter are equilibrating between the monomeric G-quadruplex and duplex at pH 5 but prefer to form duplex at higher pH. When these oligonucleotides were incubated with PIPER, P-TRIS, or $TmPyP_4$, the results are similar to those of the telomeric sequence in which PIPER and P-TRIS can induce G-quadruplex formation, while $TmPyP_4$ prefers binding to the duplex. The influence of pH is also similar to that for the telomeric sequence. Moreover, PIPER can induce many G-quadruplex structures, but P-TRIS can only induce the monomeric form. On the other hand, PIPER and $TmPyP_4$ can induce the dimeric G-quadruplex from this c-*MYC* sequence, but not with the telomeric sequence.

P-HIS induced the formation of the dimeric G-quadruplex in a concentration- and pH-dependent manner, but did not appear to bind to ds-DNA. This preference for dimeric G-quadruplex formation by P-HIS was not observed in the experiments with the telomeric sequence; therefore, it might reflect different G-quadruplex structures from different DNA sequences. Monomeric G-quadruplex also increased in the presence of P-HIS at pH 5, but not at pH 6 or 7. The influence of pH might be rationalized by the charged state of P-HIS. As noted above, P-HIS possesses side chains composed of carboxylic acid groups (pK_a = 2.82 and 3.43) and imidazole groups (pK_a = 6.25 and 6.85). At the pHs employed (pH 5–7), the carboxylic acid groups are mostly deprotonated (negatively charged), while the imidazole groups

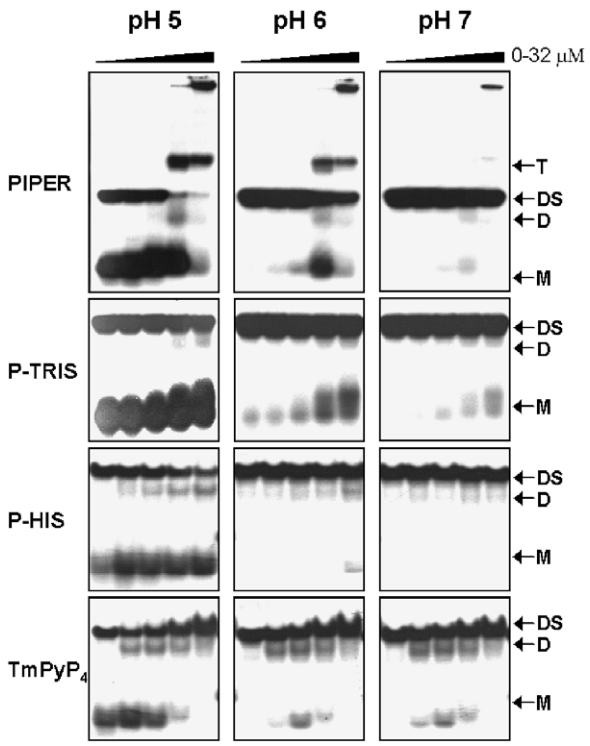

Figure 6. Duplex/G-quadruplex competition assay with c-*MYC* sequence. The duplex (HNE27G/C, 2 μM) was mixed with PIPER, P-TRIS, P-HIS, or $TmPyP_4$ (0, 4, 8, 16, and 32 μM) in 10 mM buffer containing 100 mM KCl (pH 5 = potassium acetate buffer, pH 6–7 = potassium phosphate buffer). The mixture was heated to 95 °C for 5 min and then incubated at 55 °C for 10 h in a thermocycler before cooling to 4 °C. The samples were separated by electrophoresis at 4 °C in a 16% non-denaturing polyacrylamide gel containing 50 mM KCl in both gel and electrophoresis buffer. Bands are identified as monomeric G-quadruplex (M), dimeric G-quadruplex (D), double-stranded DNA (DS), and tetrameric G-quadruplex DNA (T). Only the results from the radiolabeled G-strand are shown.

are more protonated at lower pH than at higher pH, rendering them with varying degrees of positive charge. The overall net charges at the different pHs are: pH 5 = −0.03, pH 6 = −0.45, and pH 7 = −1.49.[6] Therefore, the formation of G-quadruplex likely depends on the charged state of P-HIS; the more positive charge on P-HIS, the better the formation of dimeric G-quadruplex.

From Figure 6, P-HIS seems to induce the formation of both monomeric G-quadruplex and dimeric G-quadruplex at pH 5. Since this oligonucleotide preferentially forms monomeric G-quadruplex at pH 5 even in the presence of the complementary strand, we wondered whether the monomeric G-quadruplex was bound and stabilized by P-HIS. Lane C in Figure 7 shows that P-HIS facilitates the formation of dimeric G-quadruplex (D) and might bind to monomeric G-quadruplex (M). However, when we added the complementary strand, the intensity of the M-band gradually decreased with time, while the intensity of the D-band stayed the same. We conclude from these observations that P-HIS at pH 5 induces the formation of dimeric G-quadruplex from this oligonucleotide.

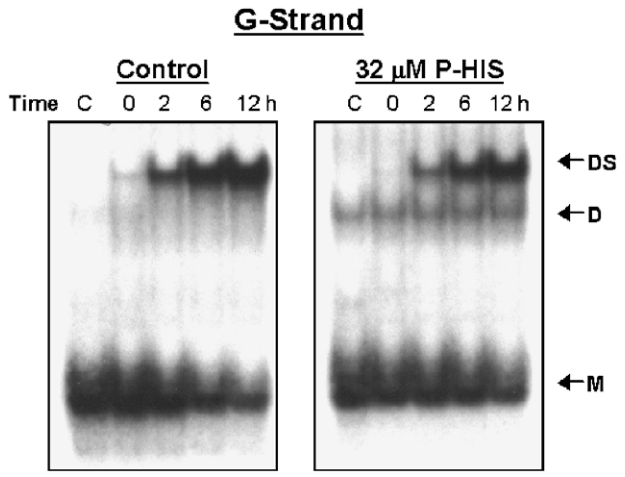

Figure 7. Stabilization of the dimeric G-quadruplex by P-HIS. The 120 μL reaction mixtures containing oligonucleotide NHE27G (2 μM, 120 kcpm), with 32 μM P-HIS (32 μM P-HIS panel) or without (control panel), were incubated at 55 °C for 10 h in buffer, pH 5, containing 10 mM potassium acetate and 100 mM KCl. Aliquots of 20 μL were taken from both samples (lane C) before an equimolar amount of complementary strand (NHE27C) was added. The samples were incubated further at 37 °C and the 20 μL aliquots were taken at the indicated times. All aliquots were then separated by 16% non-denaturing polyacrylamide gel electrophoresis at 4 °C (both gel and TBE buffer were supplemented with 50 mM KCl). Bands are identified as monomer (M), dimer (D), and double stranded DNA (DS).

In this study, we investigated the role of the pH on the G-quadruplex binding selectivity of three new perylene derivatives and compared their selectivity to that of two well-studied G-quadruplex ligands: PIPER and $TmPyP_4$. In the case of PIPER, we found that pH affects the solubility of this compound, with little change in its charge within the range of pHs used. The availability of PIPER in solution then dictates the type of G-quadruplex induced by PIPER, but fails to influence its binding selectivity. P-TRIS follows the same trend as PIPER, although the effects appear less pronounced. For $TmPyP_4$, the influence of pH is minimal.

P-HIS is a molecule that is able to change gradually from neutral state at pH ~5 to about −1.5 at pH 7 and to the fully −2 charge at pH ~8. This molecule offers us the chance to study the influence of charge on G-quadruplex binding selectivity without the concern of structural differences of the side chains. In its neutral state, P-HIS induces the formation of dimeric G-quadruplex from the G-rich strand of c-*MYC* promoter, but not with the G-rich strand of the telomeric sequence. However, in its negatively charged state, P-HIS behaves like P-GLU, another negatively charged perylene; both exhibit no interaction with any form of DNA. We cannot, however, draw a general conclusion that negatively charged side chains prevent perylenes from binding to G-quadruplex, given that the two previously reported negatively charged perylenes, perylene-EDTA and Tel12, were observed to bind preferentially to G-quadruplex DNA.[4d,4f] The main difference between those two derivatives, besides having different side chains, is their solubility. P-GLU and negatively charged P-HIS are highly soluble, while the latter two compounds aggregate. The branched structures of P-GLU and P-HIS, in combination with their negative charges, might prevent them from self-aggregating and DNA binding.

We have also demonstrated that the duplex/G-quadruplex competition assay can be used to test G-quadruplex ligands for their DNA binding selectivity. The assay can identify the type of G-quadruplex structures induced by the ligands or whether the ligands bind to duplex DNA. For example, $TmPyP_4$ can bind monomeric G-quadruplex but fail to induce G-quadruplex formation when the complementary strand is present. Moreover, the shift of the duplex DNA shows that $TmPyP_4$ binds preferentially to duplex DNA. Although it is well known that $TmPyP_4$ binds to duplex DNA,[9] as well as being a G-quadruplex ligand,[2d,8] there is no direct comparison of DNA binding selectivity. Our studies show that $TmPyP_4$ preferentially binds to duplex DNA rather than G-quadruplex, while the perylenes prefer G-quadruplex to duplex DNA.

Ideal G-quadruplex ligands should bind selectively to their target with little interaction with duplex DNA. The perylene core seems to have a binding preference for the G-quadruplex structure. Variations in the side chains of perylene derivatives might enable them to induce and/or stabilize certain G-quadruplex structures. Whether to inhibit telomerase or other potential targets, such as c-*MYC* gene, the ability to bind selectively will be critical for any successful anticancer agent. As such, the systematic data provided here regarding the role of structure/charge of the side chains of perylene derivatives should prove useful for the further development of selective perylene-based G-quadruplex ligands.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.04.078.

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5. The synthesis and characterization of the perylene derivatives are described in the Supplementary data S2–S7. The solubility profiles of these perylenes in buffer, pH 5–9, are shown in Supplementary data S8.
6. Calculated by the pI plugin of MarvinSketch 4.0.1, ChemAxon, Budapest, Hungary. www.chemaxon.com/products.html.
7. The preparation of DNA substrates for spectrophotometric studies is described in Supplementary data S8.
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